Inhibition of Multidrug-Resistant *Acinetobacter baumannii* by Non-Viral Expression of hCAP-18 in a Bioengineered Human Skin Tissue

Centanni, J.M.[1],Thomas-Virnig, C.L., Ph.D[1],Johnston, C.E.[1], He, L.K., M.D.[2], Schlosser, S.J.[3], Van Winkle, K.F.[1], Chen, R.[3], Gibson, A.L.[3], Szilagyi, A.[2], Li, L., Ph.D[3], Shankar, R., Ph.D[2], Allen-Hoffmann, B.L., Ph.D[3]

[1] Stratatech Corporation, Madison, WI, USA, [2]Loyola University Medical Center, Maywood, IL, USA, [3]University of Wisconsin, Madison, WI, USA.

Stratatech
Excellence in Regenerative Medicine

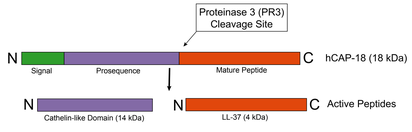
Abstract

Purpose:
Bacterial contamination is the most common reason for impairment of wound healing. During the healing response, keratinocytes produce host defense peptides (HDPs) that have antimicrobial activity against a diverse set of pathogens. The goal of this study is to genetically engineer a human skin tissue to express the human cathelicidin HDP (ExpressGraft*Enhance*) that could ultimately be applied to burns and ulcers to counteract bacterial contamination and prevent infection.
Methods:
A non-viral vector containing the human cathelicidin (hCAP-18) cDNA was used to express cathelicidin (hCAP-18 and the mature peptide LL-37) in the NIKS® keratinocyte cell line. Clonal isolates of stably-transfected cells were isolated. To assess antimicrobial activity in vitro, topical extracts from ExpressGraft*Enhance* tissue were incubated with medium containing Staphylococcus carnosus. In vivo antimicrobial studies evaluated ExpressGraft*Enhance* in a murine burn infection model in which full-thickness scald burns were inoculated with A. baumannii. NIKS® and ExpressGraft*Enhance* tissues were grafted on these wounds after the eschar was removed. Samples were harvested at 72 hrs for quantitative bacteriological cultures.
Results:
ExpressGraft*Enhance* expresses approximately 140-fold more hCAP-18/LL-37 than unmodified NIKS® tissue and possesses key histological features of normal epidermis. Peptides extracted from the surface of ExpressGraft*Enhance* inhibited growth of S. carnosus by over 78% as compared to unmodified NIKS® tissue, confirming the antimicrobial properties of the ExpressGraft*Enhance*. Moreover, in an in vivo infected burn wound model, this tissue results in a two log reduction in a clinical isolate of multidrug-resistant A. baumannii.

Objectives

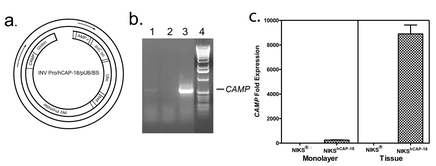
To produce a genetically modified skin substitute tissue that expresses elevated levels of the HDP, cathelicidin, and demonstrates enhanced antimicrobial activity both *in vitro* and *in vivo*.

Cathelicidin (hCAP-18) Processing



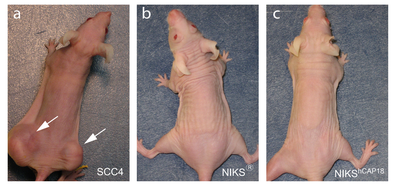
Results

Figure 1: Construction of mammalian cell expression vector, and screening of NIKS$^{hCAP-18}$ clones by semi-quantitative PCR and quantitative PCR.



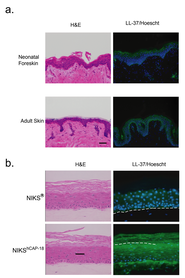
(a) Diagram of human *CAMP (hCAP-18)* expression vector.
(b) RT-PCR screen for *CAMP* transgene expression. The forward primer was designed to anneal to the *CAMP* coding region and the reverse primer was designed to anneal to the rabbit ß-globin poly A fragment of the vector. Since one primer anneals to the vector, this primer set did not amplify endogenous *CAMP* mRNA.
Lane 1: Clone with low expression of *CAMP*.
Lane 2: NIKS® cells exhibiting no *CAMP* mRNA.
Lane 3: Clone with high expression of *CAMP*.
Lane 4: 1 kb molecular weight marker
(c) Representative qPCR displaying fold expression of CAMP in monolayer and tissue formats. Monolayer clone contains ~250 fold more expression while the same clone in fully stratified tissues contains almost 9,000 fold more expression than the unmodified NIKS® control. Bars represent mean + SEM.

Figure 2: NIKS$^{hCAP-18}$ clones do not form tumors *in vivo*



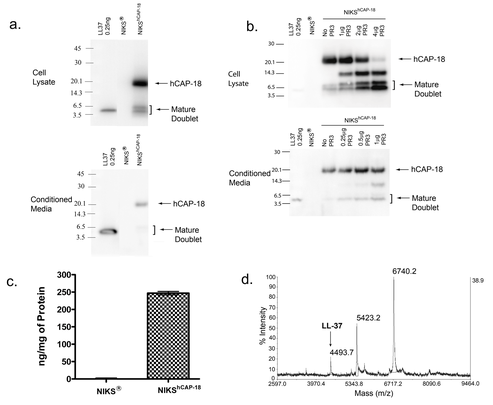
(a) Representative SCC4 injected mouse at 8 weeks.
(b) Representative NIKS® injected mouse at 12 weeks.
(c) Representative NIKS^{hCAP18} injected mouse 12 weeks.
Cells were injected into the flanks of athymic nude mice. SCC4 cells were used as a positive control for tumor production. NIKS® keratinocytes and media-only were used as a negative control. Five athymic nude mice were injected for each cell type as well as the media-only control (data not shown). SCC4 injected mice were euthanized after 8 weeks due to tumor size. After twelve weeks, NIKS®, NIKS$^{hCAP-18}$ cells as well as media-only controls did not produce tumors in athymic nude mice. Tumors (white arrows) formed only in mice injected with SCC4 positve control cells.

Figure 3: NIKS$^{hCAP-18}$ forms properly structured skin tissue and produces high amounts of hCAP-18/LL-37 that localizes to both the epidermis and dermis.



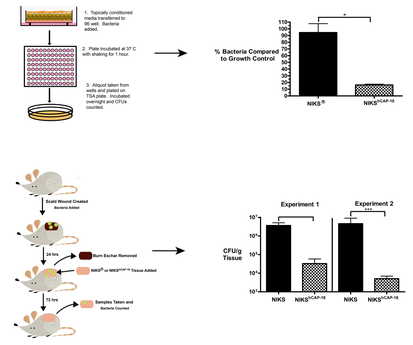
(a) Neonatal foreskin and adult skin tissue for comparison to engineered tissue.
(b) NIKS® tissue and NIKS$^{hCAP-18}$ tissue.
Left: Hemotoxylin and Eosin stained representative tissue sections. NIKS® has previously been established to form normal skin tissue including basal, spinous, granular and cornified layers. A high expressing hCAP-18 clone is indistinguishable from NIKS® tissue. Right: LL-37 (green) and Hoescht nuclei staining (blue). NIKS® tissue displays a low level of hCAP-18/LL-37 in the epidermis. NIKS$^{hCAP-18}$ produces high levels of hCAP-18/LL-37 that is found in all layers of the epidermis as well as in the dermis suggesting that the hCAP-18/LL-37 is being secreted outside of the epithelial layers. Bar equals 50µm

Figure 4: NIKS® expressing hCAP-18 produces the full length cathelicidin as well as a mature doublet form.



(a) Top immunoblot: Cell lysate from NIKS® and NIKS$^{hCAP-18}$ tissues. hCAP-18 (~18 KD) as well as a mature doublet (4-6 KD) can be detected in NIKS$^{hCAP-18}$ tissue. Bottom immunoblot: Conditioned media from NIKS® and NIKS$^{hCAP-18}$ tissues. Immunoblot analysis detects the ~18kD hCAP-18 in both NIKS$^{hCAP-18}$ cell lysate and conditioned media. Both blots were cropped to exclude duplicate samples.
(b) Top immunoblot: Cell lysate from day 15 tissue. Bottom immunoblot: 24 hr conditioned media from day 15 tissue. Proteolysis with the PR3 enzyme commonly found in wounds[3] cleaves hCAP-18 to mature forms in both cell lysate and conditioned media from NIKS$^{hCAP-18}$ tissues.
(c) ELISA analysis of tissue lysates indicates that 139 fold more mature hCAP-18/LL-37 is produced by NIKS$^{hCAP-18}$ than in the NIKS®. Bars represent mean + SD.
(d) Representative MALDI TOF/TOF mass spectrometry detects a putative LL-37 signal at m/z 4493 in the NIKS$^{hCAP-18}$ tissue.

Figure 5: Tissue created from NIKS$^{hCAP-18}$ significantly reduces the concentration of *Staphylococcus carnosus in vitro* and a clinical isolate of the pathogenic bacterium, *Acinetobacter baumannii*, in an *in vivo* model of infected burn wounds.



(a) Percent inhibition of *S. carnosus* growth for NIKS$^{hCAP-18}$ tissue as compared to NIKS® tissue *in vitro*. * represents significant (P value=0.0039) difference from the control NIKS® using an unpaired t test.
(b) Left: First *In vivo* experiment harvesting the wound bed. Right: Second *In vivo* experiment harvesting the wound bed. CFU/g of bacteria counted after 72 hrs exposure to *A. baumannii*. NIKS$^{hCAP-18}$ reduced the *A. baumannii* concentration by 2 logs. ** represents significant (P value=0.0286) difference of NIKS$^{hCAP-18}$ (n=4) from the control NIKS® (n=4) using the Mann Whitney rank sum test. *** represents significant (P value=0.0127) difference of NIKS$^{hCAP-18}$ (n=6) from the control NIKS® (n=8) using the Mann Whitney rank sum test. Bars represent mean + SEM.

Summary

* A stably transfected clonal population of cells expressing hCAP-18 was generated using non-viral methods.

* NIKS^{hCAP18} cells undergo normal squamous differentiation to produce a skin substitute possessing all the normal layers of human skin.

* The NIKS^{hCAP18} tissue expresses approximately 140-fold more hCAP-18/LL-37 protein than control tissues.

* Application of the NIKS^{hCAP18} tissue results in a 2 log inhibition of the multidrug-resistant pathogenic bacterium, *A. baumannii*, in a burn wound bed *in vivo*.

Conclusions

Non-viral genetic engineering of ExpressGraft*Enhance* alleviates concerns of safety and heterogeneity associated with viral transfection. The application of ExpressGraft*Enhance* to cutaneous wounds will not only provide temporary epithelial coverage and growth factors similar to currently available skin substitute products, but our studies provide compelling evidence that ExpressGraft*Enhance* is able to combat multi-drug resistant nosocomial strains that are of great concern to the medical community.

Acknowledgements

We thank Dr. Allen Comer and Dr. Barry Steiglitz for their editorial assistance with the completion of this work in manuscript form, Dr. Jen Eickhoff for assistance with statistical analysis and Satoshi Kinoshita for the processing of histological samples. This work was supported by the National Institutes of Health SBIR Grant NIH/NIDDK (Grant number R44 DK069924).

References

1. **Durr, UH, Sudheendra, US, and Ramamoorthy, A (2006). LL-37, the only human member of the cathelicidin family of antimicrobial peptides. Biochem Biophys Acta 1758: 1408-1425.**
2. **Allen-Hoffmann, BL, Schlosser, SJ, Ivarie, CA, Sattler, CA, Meisner, LF, and O'Connor, SL (2000). Normal growth and differentiation in a spontaneously immortalized near-diploid human keratinocyte cell line, NIKS. J Invest Dermatol 114: 444-455.**
3. **Sorensen, OE, et al. (2001). Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. Blood 97: 3951-3959.**